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# Intracellular calcium regulates agrin-induced acetylcholine receptor clustering

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# Intracellular Calcium Regulates Agrin-Induced Acetylcholine Receptor Clustering

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Agrin is an extracellular matrix protein that directs neuromuscular junction formation. Early signal transduction events in agrin-mediated postsynaptic differentiation include activation of a receptor tyrosine kinase and phosphorylation of acetylcholine receptors (AChRs), but later steps in this pathway are unknown. Here, we have investigated the role of intracellular calcium in agrin-induced AChR clustering on cultured myotubes. Clamping intracellular calcium levels by loading with the fast chelator BAPTA inhibited agrin-induced AChR aggregation. In addition, preexisting AChR aggregates dispersed under these conditions, indicating that the maintenance of AChR clusters is similarly dependent on intracellular calcium fluxes. The decrease in AChR clusters in BAPTA-loaded cells was dose-dependent and reversible, and no change in the number or mobility of AChRs was observed. Clamping intracellular calcium did not block agrin-induced tyrosine phosphorylation of the AChR $\beta$-subunit, indicating that intracellular calcium fluxes are likely to act downstream from or parallel to AChR phosphorylation. Finally, the targets of the intracellular calcium are likely to be close to the calcium source, since agrin-induced AChR clustering was unaffected in cells loaded with EGTA, a slower-binding calcium chelator. These findings distinguish a novel step in the signal transduction mechanism of agrin and raise the possibility that the pathways mediating agrin- and activity-driven changes in synaptic architecture could intersect at the level of intracellular calcium fluxes.

*Key words: agrin; intracellular calcium; AChR phosphorylation; neuromuscular junction; synaptogenesis; postsynaptic differentiation*

Synapses throughout the nervous system are characterized by high concentrations of neurotransmitter receptors in the postsynaptic apparatus (Fertuck and Salpeter, 1976; Triller et al., 1985; Jacob et al., 1986; Nusser et al., 1994). Such dense accumulations of receptors are necessary for efficient synaptic transmission. Regulation of receptor number in the postsynaptic membrane is a hallmark of synaptic development, and is likely to be an important element underlying synaptic plasticity during learning and memory (Bailey and Kandel, 1993; Weiler et al., 1995).

Agrin plays a pivotal role in synaptic differentiation at the neuromuscular junction (Hall and Sanes, 1993; Bowe and Fallon, 1995). Here, agrin secreted by motor neurons activates a receptor tyrosine kinase, MuSK, to trigger synapse formation. Mutant mice lacking either agrin or MuSK display three major abnormalities: grossly defective presynaptic and postsynaptic differentiation, and a failure in synapse selective transcription (De Chiara et al., 1996; Gautam et al., 1996). The signaling pathways between MuSK activation and these three endpoints must diverge, but in ways that currently are not understood (Gautam et al., 1995; Wells and Fallon, 1996; Apel et al., 1997).

The best-characterized branch of the agrin signaling pathway leads to the differentiation of the postsynaptic apparatus. Agrin secreted from the nerve terminal induces the aggregation of acetylcholine receptors (AChRs) and a host of other postsynaptic molecules on the muscle cell surface, including the dystrophin/utrophin-associated protein complex (Campanelli et al., 1994). The binding of agrin to a MuSK-containing complex is the first known step, with activation of the kinase occurring within minutes of agrin addition (Glass et al., 1996). Increased tyrosine phosphorylation of the AChR $\beta$-subunit is detected ~30 min later. The bench mark biological activity of agrin, the clustering of AChRs, manifests ~2 hr after agrin addition (Wallace, 1988; Nastuk et al., 1991). The maximal number of AChR clusters and level of AChR phosphorylation are achieved ~4 hr later (Wallace et al., 1991; Nastuk and Fallon, 1993).

In addition to its role in initiating postsynaptic apparatus formation, agrin also seems likely to be important for synaptic maturation, maintenance, and plasticity. Two lines of evidence point to a longer-term action in the agrin signaling pathway. First, agrin-induced AChR clusters continue to mature for at least 1 d after agrin addition *in vitro*. The AChR clusters become larger and more stable, and cytoskeletal and basal lamina elements, including agrin synthesized by muscle, accumulate with them (Wallace, 1988; Nitkin and Rothschild, 1990; Lieth and Fallon, 1993). These events require the ongoing action of agrin as well as new protein synthesis. Second, this continued action is also likely to require sustained MuSK activation (Glass et al., 1996). This long-term activation distinguishes MuSK from many other receptor tyrosine kinases, which are activated only transiently (Ullrich and Schlessinger, 1990).

Despite these advances in the understanding of the mechanisms of agrin's activity, many questions remain. For example, there is no direct evidence that tyrosine phosphorylation of AChRs is sufficient,

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or even necessary, for their clustering by agrin. Moreover, additional intracellular signal transduction events are likely to play a role in agrin-induced postsynaptic differentiation, but their nature is unknown. Of particular interest are elements that could be influenced by synaptic activity. Although it is well established that activity can shape synaptic architecture (Balice-Gordon and Lichtman, 1993; Kasai, 1993; Kirkwood and Bear, 1995; Koch, 1997), the interface between activity and the biochemical machinery that organizes synaptic structure is poorly understood.

In the present study we asked whether intracellular calcium fluxes participate in the agrin signaling pathway. We provide evidence that rapid calcium fluxes are required for agrin-induced AChR aggregation. Moreover, these fluxes act downstream from or parallel to AChR phosphorylation, which we demonstrate is not sufficient for AChR clustering. These findings reveal a novel step in the agrin signal transduction pathway. Intracellular calcium fluxes thus emerge as a potential locus for the integration of agrin- and activity-mediated changes in synaptic architecture.

## MATERIALS AND METHODS

*Myotube culture.* Embryonic chick myotube cultures were prepared as previously described (Nastuk et al., 1991). Briefly, myoblasts from embryonic day 11 chick embryos were dissociated and plated in medium containing MEM (Alpha Medium, Life Technologies, Gaithersburg, MD) supplemented with 2% chick embryo extract, 10% horse serum, 100 U/ml penicillin G, and 2 mM L-glutamine. For ligand-binding and AChR phosphorylation assays, cells were grown on plastic coated with 100 μg/ml gelatin (Sigma, St. Louis, MO). For AChR clustering assays, cells were grown on glass coverslips coated with 20 μg/ml poly-D-lysine (MW > 300,000; Sigma) and gelatin. Myotubes were used 3–7 d after plating.

*Drug treatment.* Stock solutions of the aminomethoxy ester of BAPTA (BAPTA-AM; Molecular Probes, Eugene OR) were prepared in DMSO (vehicle). The 10 mM BAPTA-AM stocks were stored at −20°C. Aliquots were thawed immediately before experiments and were not refrozen. BAPTA-AM or vehicle was diluted in serum-free medium (SFM) consisting of MEM (Alpha Medium, Life Technologies), 2 mM L-glutamine (Life Technologies), 0.5% bovine serum albumin (BSA), 100 U/ml penicillin G, and 5 μg/l each insulin, transferrin, and selenium (all from Sigma). To load cells with BAPTA, we incubated cells in BAPTA-AM for 1 hr 37°C (final DMSO concentration 0.5%) and then rinsed them with SFM. EGTA-AM (Molecular Probes) was prepared and used in a similar manner.

*AChR clustering assays.* Recombinant rat agrin, containing inserts of 12, 4, and 8 amino acids at the *x*, *y*, and *z* splice sites, respectively, was produced in COS cells as described previously (O'Toole et al., 1996). In some experiments, agrin purified from *Torpedo* electric organ (Cibacron Pool; Nitkin et al., 1987) was used with similar results. Native or recombinant agrin was used at a concentration of 10 U/ml in SFM. One unit is defined as the concentration of agrin at which half-maximal AChR clustering activity is observed (Godfrey et al., 1984).

Cells grown on coverslips were incubated with agrin for 4 hr at 37°C. Agrin was added immediately after BAPTA-AM treatment or 24 hr later (see *Washout*, Fig. 3). To detect AChRs, we included 1 μg/ml rhodamine-α-BTx in the final 45 min of the incubation. In some experiments myotubes were incubated simultaneously with agrin and KN-62 or K-252a (Calbiochem, La Jolla, CA) in DMSO. The 10 mM KN-62 and 1 mM K-252a stocks were stored at 4°C in the dark. The final DMSO concentration was ≤1%. Coverslips were rinsed in HEPES-buffered MEM (MEM-H, Life Technologies), fixed in methanol at −20°C for 5 min, mounted in Citifluor (Pella, Redding, CA), and viewed on a Zeiss Axioplan (Oberkochen, Germany) or a Nikon Eclipse (Tokyo, Japan) microscope. For quantitation of AChR clustering, 20–30 myotube segments (200 μm in length) were chosen randomly from two to three coverslips. AChR clusters (defined as AChR aggregates ≥4 μm in diameter) were scored under rhodamine optics (Nastuk et al., 1991).

Antibody-induced AChR microclustering was performed as described by Nastuk et al. (1991). Cells were loaded with BAPTA-AM or vehicle for 1 hr, rinsed, and then incubated with monoclonal antibody (mAb) 35 (Tzartos, 1983) for 30 min at 37°C, followed by goat anti-rat IgG (Sigma) and rhodamine-coupled α-bungarotoxin (α-BTx; Molecular Probes) for 30 min at 37°C. The distribution of AChRs was assessed visually; three coverslips were surveyed for each condition.

*Ligand-binding assays.* Binding of α-BTx and agrin to myotubes was quantitated as previously described (Bowe et al., 1994). Myotubes grown on gelatin-coated removable 96-well strips (Immulon 4, Dynatech, Chantilly, VA) were blocked for 1 hr in MEM-H with 1% BSA and 10% horse serum and incubated for 30 min with 10 nM $^{125}$I-α-BTx (10–20 μCi/μg, DuPont NEN, Boston, MA). For agrin binding, wells were incubated with agrin for 2 hr, followed by 1 μg/ml iodinated anti-agrin mAb 131 for 30 min. The mAb 131 (Hoch et al., 1994) was iodinated by using IODO-GEN (Pierce, Rockford, IL) per the manufacturer's instructions; the range of specific activities was 5–8 μCi/μg. Wells were washed in MEM-H, immersed twice in HBSS with 1% BSA and 1 mM calcium, dried, and counted. Nonspecific binding was determined by including 1 mM EGTA (in agrin-binding experiments) or 100-fold excess competing unlabeled α-BTx. In each experiment six individual wells were counted for each condition, and then the results of multiple experiments were pooled.

*Determination of AChR phosphorylation.* AChRs from cultured myotubes were purified according to the method of Wallace et al. (1991) with minor modifications. Biotinylated α-BTx (Molecular Probes) was purified on an ImmunoPure Immobilized Monomeric Avidin column (Pierce). Myotube cultures were loaded with BAPTA or vehicle, incubated for 4 hr in agrin and 0.5 μg/ml biotinylated α-BTx, washed twice in cold PBS, harvested, and centrifuged; the cell pellet was resuspended in extraction buffer containing (in mM) 5 EDTA, 5 EGTA, 20 Tris, pH 7.5, 20 glycine, 150 NaCl, 40 Na-pyrophosphate, 50 NaF, 10 Na-molybdate, 1 Na-orthovanadate, 5 benzamidine, 10 *N*-ethylmaleimide, and 1 phenylmethylsulfonyl fluoride, with 1% Triton X-100, 1 mg/ml bacitracin, and 50 μg/ml each chymostatin, pepstatin, aprotinin, leupeptin, and antipain. Samples were sonicated for 10 sec with a Branson 450 Sonifier at 70% power, incubated 15 min at 4°C, and then spun for 20 min at 3000 × *g*. Solubilized AChR-biotinylated-α-BTx complexes were incubated with streptavidin–Sepharose beads (Sigma) for 2 hr with constant mixing at 4°C. Beads were washed four times in extraction buffer containing 1 M NaCl, twice in extraction buffer lacking NaCl and Triton X-100, and eluted in SDS-PAGE sample buffer.

Isolated AChRs were electrophoresed on 5–15% gradient SDS-polyacrylamide gels and transferred to nitrocellulose. Then the blots were blocked in PBS supplemented with 1% BSA. To detect AChR α- and β-subunits, we probed blots with mAb 61 and mAb 111, respectively (Wallace et al., 1991) (generously provided by J. Lindstrom, University of Pennsylvania). In some experiments AChR δ-subunit was detected with mAb 88b (Froehner et al., 1983; Qu and Huganir, 1994) (generously provided by S. Froehner, University of North Carolina). Tyrosine-phosphorylated polypeptides were detected with anti-phosphotyrosine mAb 4G10 (Upstate Biotechnology, Lake Placid, NY). After incubation with primary antibody, blots were washed in PBS and incubated for 1 hr with rabbit anti-mouse IgG (Sigma), followed by 100,000 cpm/ml $^{125}$I-protein A (2–10 μCi/μg, DuPont NEN). Bound radioactivity was quantitated in each lane in regions of equal area with a Molecular Dynamics PhosphorImager and software. The amount of tyrosine phosphorylation that was detected was expressed relative to the amount of AChR loaded, as determined by quantitation of mAb 61 binding to the AChR α-subunit. In some experiments blots originally probed with $^{125}$I-protein A were stripped and reprobed with anti-AChR antibodies. Then bound antibodies were detected by biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) and an alkaline phosphatase-based ABC kit (Vectastain ABC; Vector Laboratories).

## RESULTS

### A rapid intracellular calcium chelator inhibits spontaneous and agrin-induced AChR clustering

Both the AChR clustering activity of agrin and its binding to the cell surface require extracellular calcium (Wallace, 1988; Nastuk et al., 1991). We used BAPTA to manipulate intracellular calcium without depleting calcium outside the cells. Myotubes were loaded with BAPTA, using its membrane-permeable non-calcium-binding AM ester (BAPTA-AM) (Tsien, 1981). Upon traversing the plasma membrane, BAPTA-AM is converted to BAPTA, which is membrane-impermeable, by intracellular esterases. BAPTA binds calcium rapidly, selectively, and with high affinity ($K_D$ ~100–180 nM), thus serving to "clamp" intracellular calcium fluxes (Stern, 1992; Roberts, 1993; Deisseroth et al., 1996).

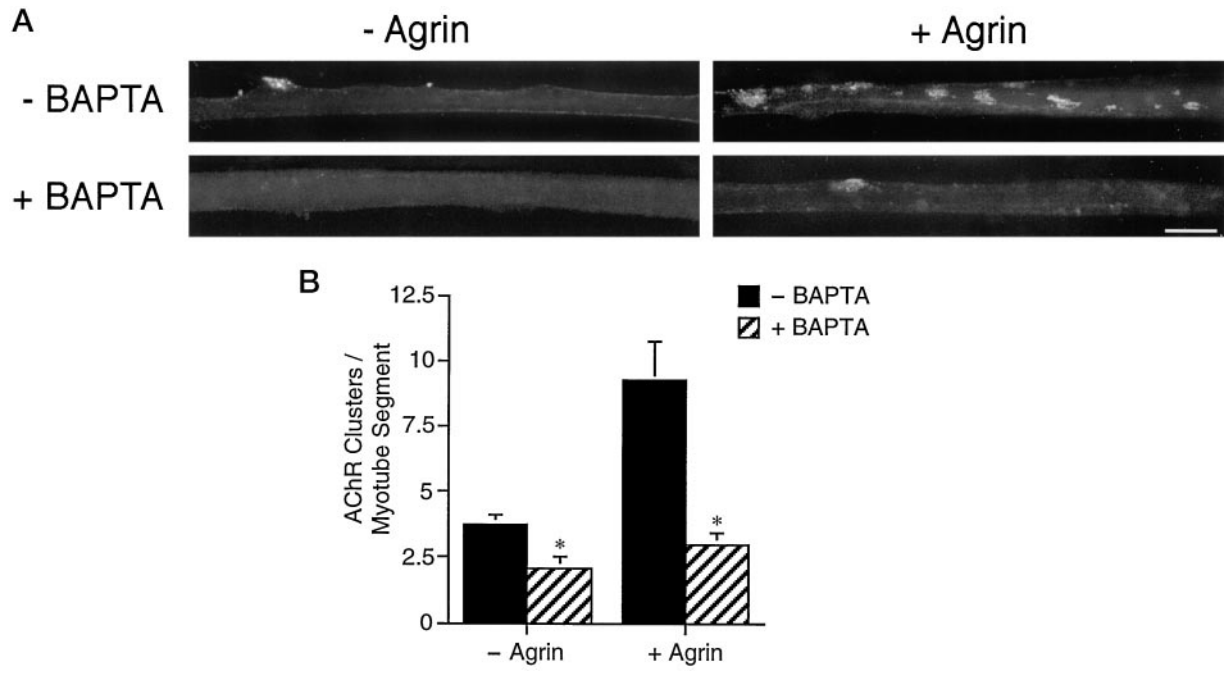


*Figure 1.* The number of spontaneous and agrin-induced AChR clusters is decreased in BAPTA-loaded cells. *A*, Myotubes were incubated with 50 $\mu$M BAPTA-AM (*bottom panels*) or vehicle only (*top panels*), washed, and incubated with or without agrin for 4 hr, as indicated. Cultures were then incubated in rhodamine-$\alpha$-BTx and examined by fluorescence microscopy to reveal the distribution of AChRs. *B*, Quantitation of AChR clusters revealed that significantly fewer spontaneous and agrin-induced clusters are observed in BAPTA-loaded cells. AChR clusters were quantitated as described in Materials and Methods. Values are mean ± SEM averaged from seven separate experiments. $^*p < 0.05$, paired Student's *t* test. Scale bar, 20 $\mu$m.

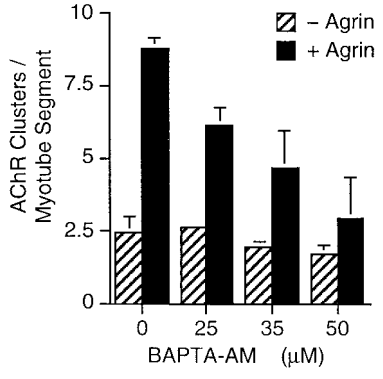


*Figure 2.* Quantitation of agrin-induced and spontaneous AChR clusters in myotubes loaded with varying concentrations of BAPTA-AM. Myotubes were incubated with the indicated concentrations of BAPTA-AM for 1 hr and then incubated in media with or without agrin for 4 hr. Data shown are from one representative experiment and are expressed as mean ± SEM. Similar results were seen in three additional experiments.

Clamping intracellular calcium inhibited AChR clustering. The number of agrin-induced AChR clusters was reduced >60% in cells loaded with 50 $\mu$M BAPTA-AM, the highest concentration tested (Fig. 1). Significant inhibition of agrin-induced AChR clustering was observed in all experiments ($n = 7$) and was dependent on the BAPTA-AM concentration used for loading (Fig. 2). The 50 $\mu$M BAPTA-AM concentration was chosen for all subsequent experiments. The number of spontaneous AChR clusters (also known as "hot spots"; Frank and Fischbach, 1979) was reduced by 40% in BAPTA-loaded cells (Fig. 1). The inhibition of spontaneous clusters was more variable than that seen for agrin-induced clusters. Although the number of spontaneous clusters decreased in BAPTA-loaded cells in all experiments ($n = 7$), the inhibition was significant in only five of them. Treatment with vehicle alone had no effect on either spontaneous or agrin-induced clusters. AChR clusters looked similar in control and BAPTA-loaded myotubes, indicating that BAPTA prevented the formation of clusters rather than causing them to form more diffusely. These results indicate that intracellular calcium fluxes are necessary for both the maintenance and the formation of AChR clusters.

We were unable to detect any deleterious effects of BAPTA loading on these cells. Myotubes loaded with BAPTA were morphologically indistinguishable from controls, as judged by phase-contrast microscopy. Moreover, the effects of drug treatment were reversible. After wash-out, the numbers of agrin-induced and spontaneous AChR clusters returned to control levels (Fig. 3).

The results presented above suggest that clamping intracellular calcium may interfere directly with the signal transduction pathway of agrin. However, it is also possible that the inhibition is attributable to indirect effects, such as altering the level of agrin-binding sites or AChRs on the cell surface. Therefore, to test these possibilities, we measured the levels of AChRs and of agrin binding. There was no statistical difference in the number of surface AChRs in BAPTA-loaded cells (102% ± 10 of control, $n = 5$; $p = 0.62$, paired Student's *t* test). Similarly, no differences in agrin binding were observed (90% ± 11 of control, $n = 4$; $p = 0.25$, paired Student's *t* test).

### The formation of antibody-induced AChR microclusters is unaffected by clamping intracellular calcium

It is possible that clamping intracellular calcium could inhibit AChR clustering by immobilizing AChRs in the myotube membrane. Such immobilization of AChRs, with concomitant inhibition of agrin-induced receptor clustering, has been reported in myotubes treated with tyrosine phosphatase inhibitors (Meier et al., 1995). To assess AChR mobility, we tested the ability of anti-

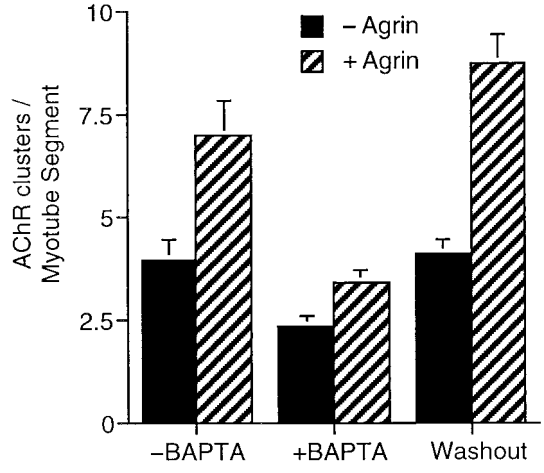


*Figure 3.* Inhibition of AChR clustering in BAPTA-loaded cells is reversible. Cells were loaded with 50 μM BAPTA-AM or vehicle for 1 hr, washed, and then incubated with agrin either immediately or 24 hr later (*Washout*). After the wash-out period, the numbers of spontaneous and agrin-induced AChR clusters returned to levels similar to vehicle-treated cells. Values are expressed as mean ± SEM from one experiment. Similar results were observed in four other experiments.

AChR antibodies to drive the formation of AChR microclusters (Nastuk et al., 1991). This manipulation is distinct from agrin-induced clustering because it relies on the direct antibody-mediated cross-linking of AChRs. Antibody-driven AChR microclustering was equally robust in both untreated and BAPTA-loaded myotubes (Fig. 4). Together with the binding data presented above, these observations support the hypothesis that intracellular calcium fluxes play a direct role in the agrin signaling pathway.

### A slower-binding calcium buffer does not inhibit agrin-induced AChR clustering

To better characterize the mode of action of calcium in the agrin signaling pathway, we tested another calcium chelator, EGTA. This chelator binds calcium with a similar affinity to BAPTA, but with a 400-fold slower on rate. As a result, calcium issuing into the cytosol can be buffered to within ~0.1 μm of the membrane in a BAPTA-loaded cell but only to ~1 μm in the presence of EGTA (Stern, 1992; Roberts, 1993; Deisseroth et al., 1996). In contrast to the results observed in BAPTA-loaded cells, the numbers of spontaneous and agrin-induced AChR clusters were unaffected in myotubes loaded with EGTA (Fig. 5). Different AM ester compounds may load into cells at different rates (Deisseroth et al., 1996). Therefore, we tested a wider range of EGTA-AM concentrations. We observed no inhibition of either agrin-induced or spontaneous AChR clusters when we used an EGTA-AM concentration ranging from 25 to 100 μM (the highest dose tested; data not shown). These results indicate that global buffering of calcium is unlikely to account for the BAPTA-mediated inhibition of AChR cluster formation. Moreover, these results demonstrate that the inhibition of AChR clustering is not the result of nonspecific side effects resulting from the use of AM ester compounds. Finally, these findings indicate that AChR clustering is likely to rely on calcium-sensitive effectors localized <1 μm from the calcium source.

One candidate effector is calcium/calmodulin-kinase II (CaM-KII). To test whether this enzyme plays a role in AChR cluster formation, we incubated myotubes with agrin in the presence of CaM-KII inhibitors (either 10–100 μM KN-62 or 0.01–10 μM K-252a). No effects on AChR clusters were observed (data not shown), suggesting that CaM-KII does not participate in the agrin signaling pathway.

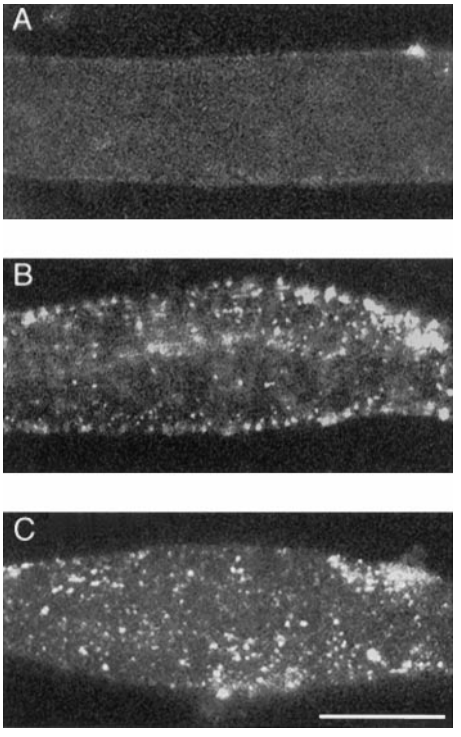


*Figure 4.* Antibody-driven AChR microclustering is unaffected in BAPTA-loaded cells. Myotubes were incubated with vehicle only (*A*, *B*) or 50 μM BAPTA-AM (*C*) and then directly incubated in buffer alone (*A*) or anti-AChR antibody mAb 35 and anti-Rat IgG for 1 hr at 37°C (*B*, *C*). The distribution of AChRs was then determined by labeling with rhodamine-α-BTx. In the absence of anti-AChR antibody incubation, AChRs were distributed diffusely on the myotube surface (*A*). Incubation with anti-AChR antibodies caused extensive AChR microclustering in both vehicle (*B*) and BAPTA-loaded (*C*) cells. Scale bar, 20 μm.

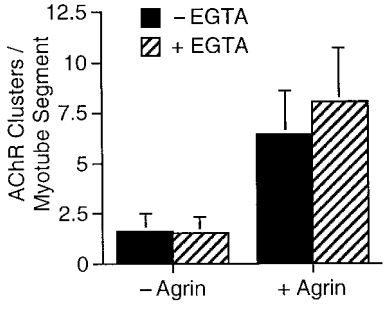


*Figure 5.* Spontaneous and agrin-induced AChR clusters in EGTA-loaded cells. Myotubes were loaded with EGTA by incubating them with 50 μM EGTA-AM for 1 hr at 37°C and then incubating them with or without agrin, as in Figure 1. The numbers of neither spontaneous nor agrin-induced AChR clusters were significantly different in EGTA-loaded, as compared with vehicle-loaded cells. Mean ± SEM from one representative experiment; $p = 0.392$ and 0.360 for spontaneous and agrin-induced clusters, respectively, Student's $t$ test. Similar results were obtained by using cells loaded with 25 or 100 μM EGTA-AM.

### Clamping intracellular calcium does not perturb agrin-induced AChR phosphorylation

We next wished to position intracellular calcium fluxes relative to known events in the agrin signaling pathway. As discussed above,

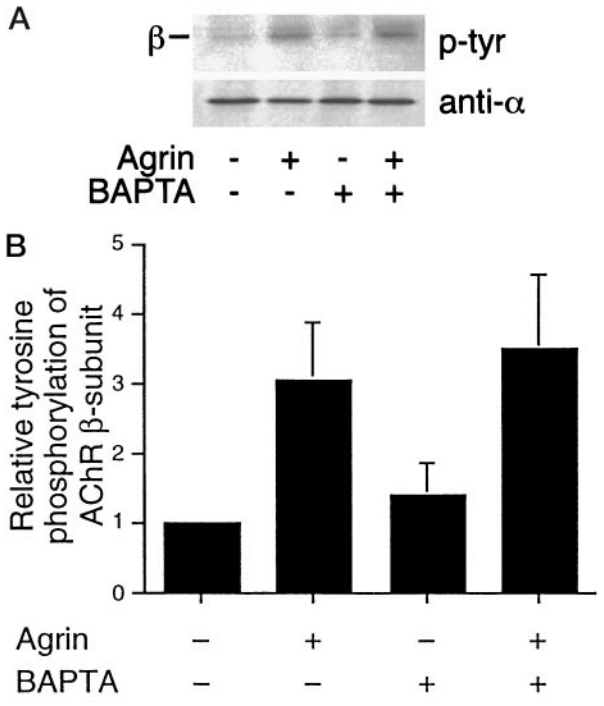


*Figure 6.* Agrin-induced tyrosine phosphorylation of AChR β-subunit in BAPTA-loaded cells. Myotubes were loaded with BAPTA or vehicle and then incubated with or without agrin for 4 hr, as indicated. Surface AChRs were affinity-purified with biotinylated α-BTx and separated by SDS-PAGE. *A*, Immunoblots were probed with mAb 4G10 to visualize tyrosine phosphorylated proteins (*p-tyr*) or mAb 61 to visualize the AChR α-subunit (*anti-α*). In parallel blots, mAb 124 was used to identify the AChR β-subunit (data not shown). Agrin induced tyrosine phosphorylation of AChR β-subunit in both the presence and absence of BAPTA. A second polypeptide of slightly slower mobility was phosphorylated also. This polypeptide was identified tentatively as AChR δ-subunit, on the basis of immunoreactivity with mAb 88b (data not shown) and previous reports showing that agrin also induces the phosphorylation of this subunit (Qu and Huganir, 1994). *B*, Phosphotyrosine levels of the AChR β-subunit were measured and expressed relative to total AChR levels, as described in Materials and Methods. Results were derived from three separate experiments, each normalized to untreated controls. Agrin-induced AChR β-subunit tyrosine phosphorylation was equivalent in vehicle, as compared with BAPTA-loaded cells. The basal level of AChR β-subunit tyrosine phosphorylation was also not significantly different in BAPTA-loaded cells ($p > 0.05$; Newman–Keuls multiple comparison test, after repeated measures ANOVA of three separate experiments; data not normalized).

early steps in this pathway include the agrin-induced activation of the receptor tyrosine kinase MuSK, followed by the tyrosine phosphorylation of AChR β-subunits (Wallace et al., 1991; Glass et al., 1996). We therefore assessed AChR phosphorylation in BAPTA-loaded cells. Phosphorylation levels of AChRs were assayed 4 hr after agrin addition, the time at which AChR clustering was assessed in the above experiments and at which maximal agrin-induced tyrosine phosphorylation is achieved (Wallace et al., 1991). As shown in Figure 6*A*, agrin induced the tyrosine phosphorylation of AChRs in both BAPTA- and vehicle-loaded cells. Quantitation of phosphotyrosine levels indicated that BAPTA loading did not significantly change agrin-induced AChR β-subunit phosphorylation (Fig. 6*B*). Further, BAPTA treatment did not alter the basal level of AChR phosphorylation in these myotubes. These results demonstrate that tyrosine phosphorylation of AChR β-subunits is not sufficient to induce their clustering and indicate that intracellular calcium fluxes act downstream or parallel to AChR phosphorylation.

## DISCUSSION

The goal of this study was to test the role of intracellular calcium in the agrin signaling pathway. Our findings indicate that locally acting intracellular calcium fluxes are necessary for agrin-induced AChR clustering. The calcium-sensitive step or steps are downstream of, or parallel to, agrin-induced tyrosine phosphorylation of AChRs. We also show that agrin-induced AChR phosphorylation is not sufficient for receptor aggregation.

We used BAPTA to buffer intracellular calcium. This compound was designed by Tsien (1981) to bind calcium with high selectivity, affinity, and speed. The efficacy and specificity of this drug have been documented in numerous studies (Stern, 1992; Roberts, 1993). For example, BAPTA has been used to probe rapid calcium-signaling events mediating exocytosis (Tsien, 1981; Penner and Neher, 1988; Adler et al., 1991). Loaded via its AM-ester form, this compound has been used to manipulate calcium-activated potassium channels (Robitaille et al., 1993), to distinguish among classes of evoked EPSPs (Cummings et al., 1996), and to probe calcium transients regulating different aspects of neuronal differentiation (Gu and Spitzer, 1995).

Several lines of evidence indicate that the clamping of intracellular calcium by BAPTA perturbs a step in the signaling pathway of agrin, rather than working via indirect mechanisms. We found no evidence that BAPTA-AM or BAPTA caused toxicity in these studies. The treated myotubes were indistinguishable from controls, as judged by phase-contrast microscopy, and the effects of BAPTA were reversible (see Fig. 3). The levels of surface agrin-binding sites and AChRs were unchanged in the treated cells. It should be noted that although we used the highly active, MuSK-activating isoform of agrin in these assays (agrin 4, 8; Glass et al., 1996), it is likely that a substantial portion of the observed binding was attributable to interaction with dystroglycan on the cell surface (Bowe et al., 1994; O'Toole et al., 1996). The normal levels of both basal and agrin-induced AChR tyrosine phosphorylation observed in BAPTA-treated myotubes also indicate that basic cell functions were uncompromised and further suggest that the level of MuSK on the cell surface is not altered substantially under these conditions. AChRs in the membrane remained mobile, as judged by the robust AChR microclustering driven by anti-AChR antibodies (see Fig. 4). However, the possibility remains that BAPTA causes relatively small changes in receptor mobility that could be beyond the sensitivity of this assay. Finally, nonspecific side effects stemming from the use of AM esters are unlikely, because loading cells with EGTA-AM had no effect on spontaneous or agrin-induced AChR clusters (see Fig. 5).

The results presented here provide new insights about the roles of MuSK activation and AChR tyrosine phosphorylation in agrin-induced AChR clustering, and their places in the signaling pathway. Activation of the MuSK receptor complex by agrin is essential for synaptic differentiation (De Chiara et al., 1996). Subsequent to this step, and dependent on MuSK activation (Apel et al., 1997), is the tyrosine phosphorylation of AChR β-subunits. Because AChR tyrosine phosphorylation proceeds normally in BAPTA-loaded cells (see Fig. 6), neither MuSK activation nor the phosphorylation of AChR β-subunit is sufficient for agrin-induced AChR aggregation. Therefore, additional components of the agrin signaling pathway, at least some of which rely on intracellular calcium fluxes, must come into play to achieve and to maintain postsynaptic differentiation.

Our studies show clearly that agrin-induced tyrosine phosphorylation of the AChR β-subunit is not sufficient for clustering

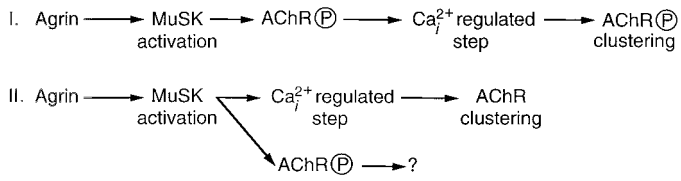


*Figure 7.* Two models of the signaling pathway of agrin. The first step in the signaling pathway is the activation of the MuSK receptor tyrosine kinase by agrin. The subsequent tyrosine phosphorylation of the AChR is dependent on MuSK activation. The results presented here are consistent with two models. (1) The intracellular calcium-regulated step occurs downstream of AChR phosphorylation. (2) The AChR phosphorylation is on a pathway parallel to (but not necessarily required for) agrin-induced AChR clustering. See Discussion for details.

AChRs. However, it is not known if this phosphorylation is necessary for agrin-induced AChR clustering. Accordingly, we present two possible models for the agrin signaling pathway (Fig. 7). In one model, the calcium-dependent step occurs downstream of agrin-induced AChR phosphorylation. In the second, agrin-induced AChR phosphorylation is a step in a parallel pathway that does not play a direct role in clustering.

Calcium is likely to play a role at both extracellular and intracellular loci during agrin-induced postsynaptic differentiation. Extracellular calcium is necessary for nerve- and agrin-induced AChR clustering (Henderson et al., 1984; Wallace, 1988). Removal of extracellular calcium destabilizes AChR clusters (Connolly, 1984; Wallace, 1988; Caroni et al., 1993; Dmytrenko and Bloch, 1993), and raising extracellular calcium can promote AChR clustering (Mook-Jung and Gordon, 1995). In addition, a large fraction of agrin binding to the cell surface and to the major agrin-binding protein on the cell, dystroglycan, is dependent on extracellular calcium (Nastuk et al., 1991; Bowe et al., 1994).

The source of the intracellular calcium transients that are necessary for AChR aggregation is not known. The requirements for extracellular calcium noted above are consistent with the possibility that the extracellular pool could be one source. Both the AChR itself and voltage-dependent calcium channels are potential conduits for such extracellular calcium influx. However, they are unlikely to be the sole sources, because neither $\alpha$-BTx nor tetrodotoxin inhibits AChR clustering induced by neurons or agrin (Anderson et al., 1977; Godfrey et al., 1984). However, calcium entering from these sources could modulate postsynaptic differentiation (see below). Alternatively, the calcium fluxes may arise from intracellular stores, such as IP3-mediated release from the endoplasmic reticulum (Verma et al., 1990).

The experiments comparing the effects of EGTA and BAPTA provide information about the location of the calcium targets relative to the sources. As a consequence of its slower-binding kinetics, EGTA, even when present at saturating concentrations, can buffer a calcium transient only to within 1–2 $\mu$m of its source. On the other hand, BAPTA is estimated to buffer calcium ions within 0.1 $\mu$m from a source (Stern, 1992; Roberts, 1993; Schweizer et al., 1995; Deisseroth et al., 1996). For example, neurotransmitter release mediated by voltage-gated calcium channels is blocked by BAPTA, but not by EGTA (Adler et al., 1991; Augustine et al., 1992). Further, BAPTA, but not EGTA, blocks activity-mediated cAMP response element binding protein (CREB) phosphorylation in hippocampal neurons (Deisseroth et al., 1996). Deisseroth and colleagues also showed that equivalent levels of intracellular EGTA and BAPTA were achieved when the loading concentration of EGTA-AM was threefold greater than that of BAPTA-AM. In the present study we observed significant inhibition of agrin-induced AChR clustering in cell loaded with 25 $\mu$M BAPTA-AM, but we observed no effects when the cells were loaded with 100 $\mu$M EGTA-AM (see Figs. 2, 5). The inability of EGTA to inhibit agrin-induced or spontaneous AChR clustering thus indicates that the sources of the calcium fluxes are likely to be close to their targets.

A possible connection between the intracellular calcium requirements observed here and the pathogenesis of muscular dystrophies deserves comment. Many varieties of muscular dystrophy, including the most prevalent forms, Duchenne and Becker, are the result of deficiencies in the dystrophin-associated protein complex. Along with clustering AChRs, agrin also induces the aggregation of many members of this complex. Moreover, altered intracellular calcium levels and calcium channel properties in dystrophic muscle have been reported (for review, see Gillis, 1996). It is also of interest that limb–girdle Muscular Dystrophy type 2A is due to a defect in calpain, a calcium-activated protease (Richard et al., 1995; van Ommen, 1995). We speculate that the organization of the dystrophin-associated protein complex involves a calcium-dependent step. Together, these observations raise the possibility that there may be links between agrin's mechanism of action and the molecular pathophysiology of muscular dystrophies.

Finally, the requirement for intracellular calcium fluxes in the AChR clustering activity of agrin presents an attractive locus for activity-mediated regulation of synaptic structure. Activity has far-reaching effects on synaptic structure and function (Balice-Gordon and Lichtman, 1993; Kasai, 1993; Kirkwood and Bear, 1995; Koch, 1997). Many of these events have been linked to changes in intracellular calcium. For example, neurotransmitter receptor synthesis in muscle is inhibited by electrical activity in a pathway initiated by calcium influx through voltage-dependent calcium channels (Huang et al., 1994). Calcium also plays a central role in the mechanisms underlying LTP and LTD at neuronal synapses (Bear and Malenka, 1994). At the neuromuscular junction such calcium fluxes could feed into the cellular and molecular machinery of the agrin signaling pathway to shape the synapse in an activity-dependent manner. It is tempting to speculate that similar mechanisms may underlie the structural plasticity of synapses in the CNS.

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